THERMAL BIOSENSOR BASED MICROMACHINED Y-CUT QUARTZ RESONATORS

A Thesis in
Electrical Engineering
by
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In this thesis, a high-sensitivity, calorimetric, point-of-care device for automated bedside urine testing based on a micromachined thermal sensor is presented. Measurements of the heat of reactions from enzymatic catalysis of glucose, urea and creatinine, were performed using glucose oxidase, urease and creatinine deiminase respectively in liquid batch testing with open air reaction chamber configuration.

For the continuous flow configuration using the integrated fluidic set-up, immobilized urease and creatinine iminohydrolase enzymes on thin (25 μm) Kapton® films are used to measure the catalytic calorimetric output as a function of concentration. The sensor design locates the active Kapton® film in close proximity to the quartz temperature sensor thereby providing an efficient heat coupling between the two. Measurements of urea concentration at room temperature using the continuous flow set-up and creatinine in open chamber setup at 37 °C are presented. Concentrations of 50 mM for urea and 100 μM for creatinine have been measured.

This thesis presents the first non-contact, continuous flow thermal sensing measurements. The unique sensor design allows for enzyme immobilized Kapton® film as an easy to swap disposable cartridge and represents a significant advancement for clinical diagnostic application.
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Chapter 1

Introduction

A micromachined calorimeter for biochemical sensing is presented in this thesis. Chapter 1 will begin with a brief description of the motivation and the goal of this thesis. This is followed by a review of some basic concepts of micromachined quartz resonator and biosensors. Finally, the chapter presents a detailed description of calorimetric biosensors.

1.1 Motivation

Acute kidney injury (AKI) occurs in 5-7% of hospitalized patients and results in a mortality rate of about 50% [1]. Serum creatinine is the traditional standard measure of kidney function. However, it is insensitive to early changes in kidney function in an acute setting. In contrast, measurements of urine creatinine may be both sensitive and early indicators of acute kidney dysfunction. Techniques for continuous measurement of urine creatinine are currently unavailable and intermittent measurements based on techniques such as the Jaffé reaction are effort intensive and limit the practical frequency of measurements that can be performed. Until now the development of creatinine sensors has focused on spectrophotometric techniques based on Jaffé reaction and electrochemical sensors [2]. In spite of the success in demonstrating high sensitivity using these methods, electrochemical sensors, in addition to issues related to enzyme-stability, have reliability concerns due to fouling of the electrodes resulting in long-term drift and variations, while spectrophotometric methods are difficult to miniaturize for continuous, in-line creatinine measurements [3].
Calorimetry is a very powerful and an effective investigative tool for analyzing biochemical reactions. Most importantly, thermal transducers can be mounted in a protected way to prevent drift in their response due to fouling of the base transducer. This endows thermal biosensors with unmatched operational stability for continuous monitoring, restricted only by the stability of the immobilized enzyme layer. The proposed Y-cut quartz resonator based sensor exhibits unmatched signal-to-noise performance and is ~250 times more sensitive than currently available temperature dependent resistors or thermocouples. This presents a unique opportunity to develop a potentially transformative, low cost, fast response, handheld sensing system for clinical diagnostic applications. In this thesis, we present the measurement results on the first noncontact thermal sensor configuration capable of continuous monitoring of kidney function based on the measurement of urinary creatinine excretion.

In summary, the following topics are addressed in this thesis:

1. Review the theory about quartz crystal resonators and biosensor
2. Enzyme immobilization protocol and implementation
3. Characterization and calibration of quartz resonator and thermal biosensor
4. Thermal biosensor measurement results of both liquid batch and continuous flow setup

1.2 Micro-machine quartz resonator

1.2.1 Piezoelectric effect

Piezoelectricity is the ability of some materials such as notably crystals and certain ceramics to generate an electric potential in response to applied mechanical stress. This
takes place in the form of separation of electric charge across the crystal lattice. If the material is not short-circuited, the developed charge induces a voltage across the material.

The piezoelectric effect is reversible in that materials exhibiting the direct piezoelectric effect (the production of electricity when stress is applied) also exhibit the converse piezoelectric effect (the production of stress and/or strain when an electric field is applied). The piezoelectric effect has found applications in high voltage applications, sensors, actuators, frequency standards, motors, and so on.

1.2.2 Quartz crystal
Quartz is the most abundant mineral in the earth’s crust. It is made up of a lattice of silica (SiO$_2$). It belongs to the rhombohedral crystal system. The ideal crystal shape is a six-sided prism terminating with six-sided pyramids at each end. Figure 1.1 depicts the various quartz crystal cuts with respect to the crystal orientation. Depending on the cutting angle, the quartz crystals show different inherent properties.

The quartz plate has many modes of vibration. There are three basic modes of vibration as illustrated in Figure 1.2.

(a) Flexure mode cuts: 5° X, NT.

(b) Extensional mode cuts: MT, GT.

(c) Shear mode:

- Face shear: CT cut, DT cut.

- Thickness shear: AT cut, BT cut, SC cut and Y-cut.
Figure 1.1: Schematic depiction of a grown quartz crystal [4].

Figure 1.2: Various Modes of operation of Quartz [5]

1.2.3 Quartz resonator

The fundamental frequency of a quartz resonator can be given by equation 1.1

$$f_0 = \frac{1}{2t} \sqrt{\frac{\mu}{\rho}}$$

(1.1)

Where, $f_0$ is the fundamental resonance frequency of the quartz plate, $t$ is the thickness, $\rho$ is the density of the quartz crystal, $\rho = 2.65 \text{ kg/m}^3$, $\mu$ is the elastic modulus associated with the elastic wave propagated in quartz. All the terms used in the above equation are
temperature dependent. Also, the temperature coefficients of the elastic modulus $\mu$ are dependent on the specific cut of quartz used. For Y-cut quartz, $\mu = 2.95 \times 10^{10} \text{ N/m}^2$.

Table 1-1 Temperature coefficient of resonance frequency for various quartz cuts [6]

<table>
<thead>
<tr>
<th>Quartz crystal cut</th>
<th>Temperature coefficient of frequency (ppm/°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AC-cut</td>
<td>20</td>
</tr>
<tr>
<td>LC-cut</td>
<td>35.4</td>
</tr>
<tr>
<td>Y-cut</td>
<td>90</td>
</tr>
<tr>
<td>SC-cut</td>
<td>-25.5</td>
</tr>
<tr>
<td>NLSC-cut</td>
<td>14</td>
</tr>
</tbody>
</table>

The Y-cut quartz which we shall use in this thesis as a sensor has a temperature sensitivity of about 90ppm / °C. We shall utilize this ability of quartz to make a quartz resonator based thermal biosensor.

The resonator can be represented by a constant clamped capacitance in parallel with an acoustic or a motional arm, which can be capacitive or inductive, which is called Butterworth-Van Dyke equivalent circuit.

For the fundamental mode, the input impedance is

$$Z_{in} = j \frac{wL_m - \frac{1}{wc_m}}{1 - w^2 C_0 L_m + \frac{C_0}{c_m}}$$

(1.2)
At series resonance, the impedance is zero, hence

\[ w_r = \frac{1}{\sqrt{L_mC_m}} \]  

(1.3)

At parallel resonance, the impedance is infinite, thus

\[ w_p = \sqrt{\frac{C_m + C_0}{L_mC_mC_0}} \]  

(1.4)

We can also relate the electromechanical coupling factor as

\[ \frac{C_m}{C_0} = \frac{8k_t^2}{N^2\pi^2} \]  

(1.5)

The motional resistance can be represented as

\[ R_m = \frac{\pi\eta\epsilon_r\epsilon_0}{8k_t^2\rho\text{Avn}_a} \]  

(1.6)
Where, \( A \) is the electrode area and \( \omega \) is the operating frequency, and \( \eta \) is the viscosity.

The values of \( C_m \) and \( L_m \) can be calculated from fundamental acoustic and electrical parameters as

\[
C_m = \frac{8}{\pi^2} k_t^2 C_0; \quad L_m = \frac{\pi^2 \nu_a}{8 w_r^2 \varepsilon_r \varepsilon_0 A k_t^2}
\]

(1.7)

The quality factor of the resonator can be calculated as

\[
Q = \frac{w_r L_m}{R_m}
\]

(1.8)

The electrical response of quartz resonator is simulated by Butterworth Van-Dyke model

![Simulated impedance spectrum from the Butterworth Van-Dyke equivalent circuit showing the resonance frequencies][1]

Figure 1.4: Simulated impedance spectrum from the Butterworth Van-Dyke equivalent circuit showing the resonance frequencies [8]

We can see two resonance frequencies at phase shift \( \varphi = 0 \), called the series resonance frequency \( (f_s) \) and the parallel resonance frequency \( (f_p) \).
The extremely high temperature sensitivity of Y-cut quartz of about \( \sim 10^{-6}/^0C \) represents two to three orders of magnitude improvement in temperature sensitivity as compared to other temperature dependent phenomena. We know that the temperature sensitivity of Y-cut quartz is about 90 ppm / ^0C. Miniaturization of the quartz resonators causes an increase in the resonance frequency and a corresponding increase in the Q-factor. This increase in the quality factor of the resonator will result in a very stable resonance frequency and reducing the noise caused in the accuracy of its measurement. The increase in the fundamental resonance frequency of the Y-cut resonator increases the per degree resonance shift obtained in direct proportion as shown in equation 1.10

\[
\Delta f = f_0 \Delta T \frac{90}{10^6}
\]

(1.10)

Where, \( \Delta f \) is the shift in the resonance frequency of the quartz resonator, \( f_0 \) is the fundamental resonance frequency and \( \Delta T \) is the change in temperature. As can be seen from the above equation, an increase in the fundamental resonance frequency causes an increase in the frequency shift per degree rise in temperature. This makes a resonator with very high resonance frequency capable of resolving and measuring small changes in temperature.
1.3 Biosensor

1.3.1 Biosensor definition

Biosensor is a device that responds to the presence of a particular analyte in a selective way through a biochemical reaction and can be used for qualitative or quantitative determination of the analyte. It incorporates a biological recognition element connected to a transducer as shown in Figure 1.5.

![Figure 1.5: Schematic representation of biosensor construction](image)

Bio-recognition elements are the key components for responding to the target analyte without interferences from others and to integrate the biological component with the selected transduction method. Depending upon the working principle, bio-recognition elements can be divided into two categories: catalysis type and binding type. For the catalysis type, the biomolecule catalyzes a change in a physicochemical parameter; while the binding type relies upon a strong binding of the analyte to the biomolecule [10].
1.3.2 Biosensor performance criteria & applications

Since biochemical sensors operate with many different detection techniques and are available in many different configurations, it is important to be able to specify their performance capabilities in a universal set of metrics. Any given sensor may be specified using several or all of the metrics listed below. However, there exists no sensor that can achieve high performance specifications on all of the listed metrics. In fact, current efforts in biochemical sensing technologies are focused on improving many of these parameters [10].

i. Sensitivity: The amount of change in a sensor’s output in response to a change in the sensor’s input over the sensor’s entire range. Sensitivity range is important to specify which concentration range is detectable and which section of this range has linear response. The lower level of this range is normally known as the detection limit.

ii. Accuracy: The degree of correctness with which a measuring system yields the “true value” of a measured quantity.

iii. Resolution: The smallest increment of change in the measured value that can be determined from the instrument’s readout scale.

iv. Precision: The difference between the instruments’ reported values during repeated measurements of the same quantity, typically determined by statistical analysis of repeated measurements.

v. Selectivity: The ability of a sensor to measure only one input parameter. Most sensors will respond mainly to one analyte, with a limited response to other similar analytes. This behavior is principally a function of the selective component.
vi. Reversibility: One of the important features of biochemical sensors is its capability to be used repeatedly. Reversibility relates to the ability of the sensor to revert back to the unperturbed specifications when the input is completely removed. Achieving high reversibility is a major challenge in biochemical sensors.

vii. Response time: Biosensors output typically requires a certain time to stabilize, which among many other factors includes the time for the biochemical reactions to attain equilibrium rate. The required time is known as response time, which can vary from a few seconds to a few minutes.

viii. Recovery time: the time required between two measurements and is related to the reversibility of the biochemical reactions described earlier.

ix. Working lifetime: the time after which the response has declined by a given percentage during continuous use.

Biosensors have been used in many fields, including clinical diagnostics, process monitoring and environmental control. Clinical diagnostic applications claim close to 92% of the biosensor market, which was about 180 billion of dollars in year 2001 [11]. Measurements of blood, gases, ions and key metabolites are highly valuable for diagnostics under critical metabolic circumstances. Glucose, urea, lactate, sodium, potassium and calcium are some of the routinely needed assays for diagnostic work. The ideal biosensor might be an implanted sensor integrated with a microprocessor controlled drug delivery system for continuous monitoring of a metabolite. Such a device would be attractive for the treatment of chronic illnesses.
1.4 Thermal biosensor

1.4.1 Calorimetry

Calorimetry is the science of measurement of the amount of heat evolved or absorbed in a chemical reaction, change of state, or formation of a solution. Since most biological reactions involving the breakdown of complex molecules are more or less always exothermic in nature, these can be investigated using calorimetric analysis.

Furthermore, calorimetric measurements by themselves are not reaction specific and thus can be used with a wide range of materials. Specificity to identify or perform a selective assay can be achieved by coating the calorimeter with specific catalysts such as enzymes, antibodies or even single stranded oligonucleotides for DNA sequence analysis [12].

When the analyte is exposed to the catalyst, the biochemical reaction begins and its evolution in terms of total amount of heat generated and kinetics is proportional to the reactants concentration as well as the rate constants of the reaction. Thus, when an exothermic biological reaction is performed on a micromachined, thermally isolated structure, the small thermal mass and good thermal isolation will allow the temperature of the structure to rise. This rise in temperature can be very accurately measured in real time using a micro thermal sensors integrated on the structure. The microcalorimeters have high sensitivity and fast response time for real-time analysis of very small quantities of analytes.
1.4.2 Enzymatic reaction kinetics

Table 1.2: Molar enthalpies of enzyme-catalyzed reactions [13]

<table>
<thead>
<tr>
<th>Reactant</th>
<th>Enzyme</th>
<th>Enthalpy $\Delta H$ (KJ/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol</td>
<td>Cholesterol oxidase</td>
<td>53</td>
</tr>
<tr>
<td>Esters</td>
<td>Chymotrypsin</td>
<td>4 - 16</td>
</tr>
<tr>
<td>Glucose</td>
<td>Glucose oxidase</td>
<td>80</td>
</tr>
<tr>
<td>Hydrogen peroxide</td>
<td>Catalase</td>
<td>100</td>
</tr>
<tr>
<td>Penicillin G</td>
<td>Penicillinase</td>
<td>67</td>
</tr>
<tr>
<td>Peptides</td>
<td>Trypsin</td>
<td>10 - 30</td>
</tr>
<tr>
<td>Starch</td>
<td>Amylase</td>
<td>8</td>
</tr>
<tr>
<td>Sucrose</td>
<td>Invertase</td>
<td>20</td>
</tr>
<tr>
<td>Urea</td>
<td>Urease</td>
<td>61</td>
</tr>
<tr>
<td>Uric acid</td>
<td>Uricase</td>
<td>49</td>
</tr>
</tbody>
</table>

Metabolic pathways are sequences of bio-reactions. Almost all cell reactions involve enzymes. Most enzyme-catalyzed reactions are exothermic, which may be used as a basis for measuring the rate of reaction and analyte concentration as shown in Table 1.2.

The rate of reaction catalyzed by a soluble enzyme can be described by *Michaelis-Menten* kinetic equation [14].

\[
A + E \overset{k_1, k_{-1}}{\rightleftharpoons} EA \overset{k_2}{\leftrightarrow} P + E
\]

(1.11)
Where E is the enzyme, A is the analyte, P is the product and EA is the enzyme-analyte complex. For a bath reaction, the balances for A and EA are written as:

\[
\frac{dA}{dt} = -k_1AE + k_{-1}EA \\
\frac{d(EA)}{dt} = k_1AE - (k_{-1} + k_2)EA
\]

(1.12)

With initial condition at \( t = 0 \): \( A = A_0 \), \( E = E_0 \), \( (EA) = 0 \).

The enzyme concentration is usually much lower than the substrate; most of enzyme is present during the reaction in the form of enzyme-analyte complex, EA. And it is possible by assuming a quasi-steady state:

\[
d(EA)/dt = 0, \ E_0 = E + (EA)
\]

(1.13)

Then EA and E concentration can be solved from equations 1.12 and 1.13:

\[
r_A = -\frac{dA}{dt} = \frac{r_{\text{max}}A}{K_M + A}
\]

(1.14)

Where \( r_A \) is the reaction rate, maximum reaction rate \( max \ r_{\text{max}} = k_2E_0 \), and the Michaelis-Menten constant \( K_M = (k_{-1} + k_2) / k_1 \). The typical value for \( K_M \) is between \( 10^{-6} \) and \( 10^{-1} \) M. The Michaelis-Menten equation exhibits three distinct regions for the reaction rate as shown in Figure 1.6. The low concentration region \( (A << K_M) \) can be approximated by first-order kinetics: \( r_A = r_{\text{max}} A / K_M \). The rate of depletion of reactant is proportional to the instantaneous concentration of sample. The reactant concentration decays exponentially: \( A = A_0 \exp (-r_{\text{max}} / K_M) \). For high substrate concentration \( (A >> K_M) \), the
relation approaches zero-order: \( r_A = r_{\text{max}} \). And the rate of reaction is independent of substrate concentration and is constant at the maximum value. In the intermediate substrate concentration range \( (0.1 \, K_M < A < 10 \, K_M) \), the full Michaelis-Menten equation must be used to guarantee an accurate \( r_A \) [13].

![Diagram showing reaction rate vs. substrate concentration for the Michaelis-Menten equation](image)

Figure 1.6: Reaction rate vs. substrate concentration for the *Michaelis-Menten* equation [13].

1.4.3 Thermal biosensor classification

There are three general types of calorimeters classified by how heat transfer takes place between the reaction vessel and the surroundings [15].

i. Heat conduction calorimetry: Heat exchanges between chamber and isothermal heat sink surrounding. Temperature changes are measured as voltage output of a thermoelectric sensor between chamber and the sink. Heat sink is kept isothermal to keep
heat transfer coefficients constant. The temperature of reaction chamber is proportional to heat generation.

ii. Isoperibol calorimetry: There is no heat transfer. The heat quantity evolved during an experiment is equal to the product between the measured temperature changes and the heat capacity of the vessel and its content.

iii. Isothermal calorimeter: Temperature of the reaction vessel is kept constant by heating or cooling.

Other ways of classification is by the mode of operation:

i. Static: isothermal, isoperibol, adiabatic

ii. Dynamic: scanning of surrounding, isoperibol scanning, adiabatic scanning and the construction mode:

- Single measurement
- Differential measurement

For each form of calorimetry, if it is used in solution condition, there are three types of reactant mixing method. These are titration, batch addition and flow mixing. In titration calorimetry, one reactant is titrated repeatedly or continuously into the other reactant. Batch addition means mixing of the total volume of both reactants in one operation. Most flow calorimeters have a reaction zone where the two flow reactants mix to form product stream. In all the cases, the temperature change or the heat produced in the system is measured.
Chapter 2

Enzyme immobilization protocol and implementation

Review of all basic enzyme immobilization techniques and the actually implemented enzyme immobilization protocol for the thermal biosensor are present in this chapter.

2.1 Enzyme immobilization concept

Today, biosensors are gradually turning into practical and important tools in medicine study, food quality control, environmental monitoring and bio-chemical research. In general concept, they can be designed to match individual analytical requests for almost any target molecule or compound that interacts specifically with a biological system. A biosensor makes use of a biological molecule that is immobilized in a proximity to a transducer to detect an analyte, and eventually transduces the chemical signal produced by the interaction into a measurable response, most often as an electronic signal [16]. To be able to reuse of the expensive biological molecule as a significant simplification of the analytical apparatus, the biomolecules can be immobilized. And in biosensor, enzymes are the most commonly used biological components.

There are numerous reasons for the preparation and use of immobilized enzymes. Along with a more convenient handling of enzyme preparations, the two main reasons why we do immobilization are how easy separation of the enzyme from the product, and the ability of reuse of the enzyme. Easy separation of the enzyme from the product simplifies enzyme applications and supports a reliable and efficient reaction technology.
Furthermore, reuse of enzymes provides cost advantages which are often an essential requirement to establish an enzyme-catalyzed process. Immobilized enzyme preparations have special properties that are governed by the properties of both the enzyme and the carrier material. The special interaction between enzyme and the carrier material provides an immobilized enzyme with distinct chemical, biochemical, mechanical and kinetic properties (Figure 2.1) [17].

![Figure 2.1: Characteristics of immobilized enzymes [17]](image)

Table 2.1 Criteria for robust immobilized enzymes [18]

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Requirement</th>
<th>Benefits</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-catalytic function</td>
<td>• Suitable particle size and shape</td>
<td>• Aid separation, easy control of the reaction</td>
</tr>
<tr>
<td></td>
<td>• Suitable mechanical properties</td>
<td>• Flexibility of reactor design</td>
</tr>
<tr>
<td></td>
<td>• Low water regain capability</td>
<td>• Easy removal of water</td>
</tr>
<tr>
<td></td>
<td>• High stability in a variety of organic solvents</td>
<td>• No change of pore radius and thus</td>
</tr>
<tr>
<td>Catalytic function</td>
<td>Fewer diffusion constraints</td>
<td></td>
</tr>
<tr>
<td>--------------------</td>
<td>-----------------------------</td>
<td></td>
</tr>
<tr>
<td>• High volume activity (U/g)</td>
<td>• High productivity and space–time yield</td>
<td></td>
</tr>
<tr>
<td>• High selectivity</td>
<td>• Fewer side reactions, easier downstream processing and separation of products, and less pollution</td>
<td></td>
</tr>
<tr>
<td>• Broad substrate specificity</td>
<td>• Tolerance of structural variation of the substrates</td>
<td></td>
</tr>
<tr>
<td>• Stability in organic solvents</td>
<td>• Shift of reaction equilibrium with the use of organic solvents</td>
<td></td>
</tr>
<tr>
<td>• Thermostability</td>
<td>• Short reaction time by increasing temperature</td>
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<td>• Operational stability</td>
<td>• Cost-effective and lower cost contribution for the product</td>
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<td>• Conformational stability</td>
<td>• Modulation of enzyme properties</td>
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<th>Immobilized enzyme</th>
<th>Economical and ecological consideration</th>
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<tr>
<td>• Recyclability</td>
<td>• Lower cost for the solid handling</td>
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<td>• Broad applicability</td>
<td>• Less environmental concern</td>
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<td>• Reproducibility</td>
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<td>• Easy and quick design</td>
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General criteria for the selection of non-catalytic and catalytic properties for robust immobilized enzymes are listed in Table 2.1.

2.2 Type of immobilization

Figure 2.2: Classification of immobilization methods [17]

Generally, there are three standard methods of enzyme immobilization: binding to a support (carrier), entrapment (encapsulation) and cross-linking immobilization. The following text is adapted from the work of Sheldon (Reference [19]):

i. Support binding: The binding force between the enzyme and the carrier can be physical, ionic, or covalent in nature. Normally, the physical bonding is too weak to hold the enzyme attached to the carrier under conditions of high reactant and product
concentrations. In contrast, the ionic binding is stronger and the covalent binding is even better, which has the advantage that the enzyme cannot be leaked from the carrier surface. Nevertheless, this also has a drawback, if the enzyme is irreversibly deactivated, both of the enzyme and the support are become unusable. Various kinds of support are available for this type of immobilization, for example they can be a synthetic resin, a biopolymer or an inorganic polymer such as silica or a zeolite.

ii. Entrapment: The entrapment of the enzyme is carried out by the inclusion of an enzyme in a polymer network such as an organic polymer or a silica sol-gel, or a membrane device such as a microcapsule. The physical restraints are normally not strong enough to avoid leakage of enzyme. Thus extra covalent attachment is often needed. There is a little bit of difficulty to distinguish between the two immobilization techniques - entrapment and support binding. In support binding technique, the enzyme is attached to a prefabricated carrier regardless of whether the enzyme is located on the external or internal surface. In contrast, in entrapment technique, the enzyme requires the synthesis of the polymeric network to entrap it inside.

iii. Cross-linking: Cross-linking immobilization technique requires the utilization of enzyme aggregates or crystals. The enzyme aggregates and enzyme crystals are bifunctional reagents which can help to prepare carrierless macro-particles. Generally, the use of a carrier unavoidably leads to the dilution of activity, and results the presence of a large percentage of non-catalytic ballast, ranging from 90% to >99%, which causes to lower space-time yields and productivities. Furthermore, loss of more than 50% native activity happens when we do immobilization of an enzyme on a
carrier, especially at high enzyme loadings. Therefore, there is a growing interest in carrier-free immobilized enzymes, such as cross-linked enzyme crystals (CLECs), and cross-linked enzyme aggregates (CLEAs). This type of immobilization offers clear advantages: highly concentrated enzyme activity in the catalyst, high stability and low production costs due to the exclusion of an additional carrier.

2.2.1 Support binding enzyme immobilization [19]

The carrier can be a synthetic organic polymer, a biopolymer or an inorganic solid.

(i) Synthetic organic polymers

For support binding immobilization using synthetic organic polymers as a support, acrylic resins such as Eupergit C are very commonly used as carrier. Eupergit C is a macroporous copolymer of N, N’-methylene-bi-(methacrylamide), allyl glycidyl ether, glycidyl methacrylate and methacrylamide. In principle, Eupergit C binds enzymes via the reaction of its oxirane moieties with the free amino groups of the enzymes to form covalent bonds (Figure 2.3). Eupergit C is greatly hydrophilic and stable, both chemically and mechanically, even with an extreme environmental pH changes. The remaining epoxy groups can be rendered inactive by coating using a variety of reagents (mercaptoethanol, ethanolamine, glycine, etc.) to prevent any undesired support - enzyme reactions. Because of the high density of oxirane groups on the surface of the Eupergit C beads, enzymes are immobilized at various sites of their structure. The high operational stability of enzymes bound to Eupergit C is based on this “multi-point-attachment” type of immobilization.
Covalent attachment to Eupergit C immobilization technique has been successfully implemented to several types of enzymes for industrial and research applications. A main disadvantage of Eupergit C is its diffusion limitations, which affect in the enzymatic kinetically controlled processes.

With many similar important characteristics to Eupergit C, Sepa beads FP-EP consist of a polymethacrylate-based resin functionalized with oxirane groups, and various other porous acrylic resins are used to immobilize enzymes via simple adsorption without covalent attachment [20].
(ii) Biopolymers

In addition to synthetic organic polymers, a variety of biopolymers, mainly water-insoluble polysaccharides such as cellulose, starch, agarose and chitosan and proteins such as gelatin and albumin have been also broadly used as carrier for immobilizing enzymes [21].

\[
\text{R} = (\text{CH}_3)_2\text{CH}, \text{CH}_3\text{SCH}_2\text{CH}_2, \text{etc}
\]

- L-specific aminoacylase from \textit{Aspergillus oryzae}
- Continuous, fixed bed (DEAE-Sephadex) operation

Figure 2.4: Tanabe aminoacylase process [19].
In fact, Tanabe process is the first industrial application of an immobilized enzyme in a biotransformation for the production of L-amino acids by resolution of racemic acylamino acids using an aminoacylase from Aspergillus oryzae (Figure 2.4). The immobilization of enzyme is carried out by ionic adsorption on DEAE-Sephadex which consists of cellulose modified with diethylaminoethyl functionalities and the process was performed in continuous operation in a fixed-bed reactor. This immobilization technique is still commonly used in the immobilization of a recombinant epoxide hydrolase from Aspergillus niger.

(iii) Inorganic solids

The next category of carriers for support binding type of immobilization is several of inorganic solids such as alumina, silica, zeolites and mesoporous silica [22]. One of the simplest and most inexpensive methods to immobilize an enzyme is by silica granulation. This method is used to formulate enzymes for detergent powders which release the enzyme into the washing liquid during washing process. Granulation technology was utilized to immobilize CaLB lipase on silica granules, by first adsorbing the lipase on silica powder followed by agglomeration.

Another example of inorganic carrier is the immobilization of chloroperoxidase (CPO) from Caldariomyces fumago on the to a mesoporous sol-gel glass to produce a higher stability towards organic solvents. The immobilized preparation was used together with free glucose oxidase which generated hydrogen peroxide in situ by aerobic oxidation of glucose. In the same way, immobilization of Mucor javanicus lipase on functionalized
Silica nanoparticles also lead to an improved thermal stability and a higher retention of activity over a wider pH range (Figure 2.5).

(iv) Hydrogels

Another carrier option of support binding immobilization technique is that enzymes can also be immobilized in natural or synthetic hydrogels or cryogels in non-aqueous solution. For the immobilization of whole cells, Polyvinyl alcohol (PVA) hydrogels formed by the freeze-thawing method have been generally used [23]. Partial drying at room temperature afforded lens-shaped hydrogels is an efficient method for preparing PVA hydrogels. Lentikats exhibits very good mechanical stability and easy separation. It has been successfully implemented for the entrapment of whole cell biocatalysts. In fact,
the immobilization of whole cells of Rhodococcus equi A4, which contains nitrile hydratase and amidase activities, has been used by hydrogels Lentikats.

Moreover, immobilization of free enzymes in PVA hydrogels can be also suitable in organic solution, such as the co-immobilization of an alcohol dehydrogenase from Lactobacillus kefir together with its co-factor, NADP, in PVA beads. The resulting immobilization was used for the enantioselective reduction of a broad range of hydrophobic prochiral ketones to the corresponding (R)-secondary alcohols in n-hexane as solvent (Figure 2.6).

\[
\text{TTN cofactor} = 10^2 - 10^3 \\
\text{STY} = 8.4 \text{ g/L/min}
\]

ADH = alcohol dehydrogenase from \textit{Lactobacillus kefir} immobilized in PVA beads (0.5 – 1 mm)

Figure 2.6: Alcohol dehydrogenase in Lentikat [19].
Besides, to retain the enzyme in a PVA hydrogel in the presence of water, the molecular weight must be increased. This immobilization technique can be done by cross-linking an (R)-oxynitrilase using a mixture of glutaraldehyde and chitosan (Figure 2.7).

Figure 2.7: Cross-linked (R)-oxynitrilase in Lentikat hydrogel [19].
Next Lentikat PVA hydrogel will entrap the cross-linked enzyme inside. The immobilized biocatalyst results no leaking in the enantioselective hydrocyanation of benzaldehyde in a biphasic aqueous buffer or organic solvent system.

### 2.2.2 Entrapment enzyme immobilization [19]

Another enzyme immobilization technique is by entrapment the enzyme in sol-gel matrices formed by hydrolytic polymerization of metal alkoxides. The morphologies of the silica sol-gels depend on the method of drying it. Nowadays, immobilization the enzyme in silica sol gels prepared by hydrolytic polymerization of tetraethoxysilane has become a popular enzyme immobilization method [24].

Enzymes can also be entrapped in polydimethylsiloxane membranes and silicone elastomers. An example of polymer-incarceration methodology for immobilizing enzymes is illustrated in Figure 2.8. The process starts by dissolving polystyrene containing hydrophilic tetraethylene glycol and glycidol moieties as pendant groups in dichloromethane, and then adding a solution of CaLB. After the decantation of the supernatant, the polymeric matrix is cross-linked by reaction of the pendant glycidol groups with a triamine at 60 °C in hexane to afford a polymer incarcerated lipase.
2.2.3 Cross-linking enzyme immobilization [19]

Early research of protein chemistry headed to the finding of cross-linking of dissolved enzymes via reaction of surface NH$_2$ groups with a bi-functional chemical reagent, such as glutaraldehyde, afforded insoluble cross-linked enzymes (CLEs) with retention of catalytic activity [25]. But in the late 1960s, carrier-bound enzymes became dominant enzyme immobilization technique to be commonly used in the industry for the next three decades.

i. Cross-linked enzyme crystals (CLEC)

The cross-linking enzyme immobilization technique using cross-linker as glutaraldehyde was first done in 1964. The use of cross-linked enzyme crystals (CLECs) as industrial
biocatalysts was pioneered by scientists at Vertex Pharmaceuticals in the early 1990s and then commercialized by Altus Biologics [26].

This immobilization method is shown to be applicable to an extensive range of enzymes. CLECs proved significantly more stable to denaturation by heat, organic solvents and proteolysis than the corresponding soluble enzyme or lyophilized powder. Their operational stability and ease of recycling, together with their high catalyst and volumetric productivities make them become ideal candidates for industrial biotransformations techniques.

ii. Cross-linked enzyme aggregates (CLEA)

One of the disadvantages of CLECs is the need to crystallize the enzyme. In contrast, it is a general fact that with an accumulation of salts, or water-miscible organic solvents, or non-ionic polymers to aqueous solutions of proteins will lead to their precipitation as physical aggregates of protein molecules, which held together by non-covalent bonding without perturbation of their tertiary structure that is without denaturation [27].

That is why that the crosslinking of these physical aggregates would render them permanently insoluble while maintaining their pre-organized superstructure and catalytic activity. CLEAs open a development of a new type of cross-linking enzyme immobilization: cross-linked enzyme aggregates (CLEA) (Figure 2.9). The CLEA technique will not only combines purification and immobilization into a single unit operation but it also does not require a highly pure enzyme.
Glutaraldehyde is one of the most commonly cross-linking re-agents of choice as it is inexpensive and readily available in commercial quantities. Besides, since cross-linking involves reaction of the amino groups of lysine residues on the external surface of the enzyme. Co-precipitate the enzyme with a polymer containing numerous free amino groups, such as poly-lysine or polyethylene imine will compensate for this lack of surface amino groups. Furthermore, to help in the formation of the CLEA, an addition of bovine serum albumin (BSA) as a “protein feeder” in the preparation of CLEAs should be made [28].

Enzymes are the common catalysts in the synthesis for most of essential chemical compounds, especially in the fine chemicals industry for the manufacture of enantiopure compounds. Therefore, enzyme immobilization is a useful tool to meet cost targets and to achieve technological advantages because of its conveniently repetitive use. However,
owing to the wide variation in the properties of the individual enzyme and the varying requirements of reaction technology for the target compounds, which of the available immobilization methods is the best will be depended on both of its specific technical requirements and the overall business framework.

2.3 Implemented enzyme immobilization methodology

Glutaraldehyde coupling is an enigma that deserves special attention as it is perhaps the mostly used method for coupling proteins to collagen, aminopropyl glass and a wide variety of amine-containing supports.

We use glutaraldehyde as a cross-linking agent to immobilize urease and creatinine deiminase to be integrated in our biosensor.

2.3.1. Chemicals

Glutaraldehyde (25% aqueous solution), γ-aminopropyltriethoxysilane (98%), bovine serum albumin (BSA), urea, urease (jack bean urease 300U/mg), creatinine, creatinine deiminase (>25U/mg), monosodium and disodium phosphates were purchased from Sigma Aldrich.

The buffer solutions were prepared by mixing 67mmol/L NaH$_2$PO$_4$ and 67mmol/L Na$_2$HPO$_4$. Buffer pH values were adjusted by changing the volume ratio of Na$_2$HPO$_4$ and NaH$_2$PO$_4$ solutions. Buffer solutions of lower molarity were prepared by dilution with distilled water.
A stock solution of urea and creatinine (1mol/L) was prepared in phosphate buffer solution, pH 6.11. Solutions with lower concentrations of urea and creatinine were prepared by subsequent dilution with phosphate buffer. The pH was measured by means of a pH-meter.

2.3.2. Substrate

For sensor fabrication, SiO$_2$ as a square surface of 15 x 30 mm$^2$ were used as substrates. We also tried plastic surfaces such as polystyrene and kapton tapes for substrate alternatives.

2.3.3. Methodology [29, 30]

Preliminarily modified surface of a transducer:

First, the substrates were thoroughly cleaned and placed in a 10% aqueous solution of γ-aminopropyltriethoxysilane. The pH of the solution was immediately adjusted with 6 mol/L HCl to pH 3.45, and the solution was placed in a water bath of 75°C for about 3h. The reaction can be schematically presented in the following way:

$$\equiv \text{Si} – \text{OH} + \text{NH}_2(C_3H_6)\text{Si(OR)}_3 \rightarrow \equiv \text{Si} – \text{O} – \text{Si(OR)}_2(C_3H_6)\text{NH}_2$$  \hspace{1cm} (2.1)

where R is an ethyl group. The derivatized substrates were washed with distilled water and heated to 100°C overnight.
Second, activation of the silanized carrier was performed via a reaction with glutaraldehyde. The reaction was carried out in a 2.5% solution of glutaraldehyde in 0.1mol/L phosphate buffer at pH 7 at room temperature for about 3 hour, followed by thoroughly washing out the excess glutaraldehyde with distilled water. The reaction can be schematically presented in the following way:

\[ \equiv \text{Si–O–Si(OR)}_2(C_3H_6)NH_2 + \text{CHO(CH}_2)_3\text{CHO} \rightarrow \]

\[ \equiv \text{Si–O–Si(OR)}_2(C_3H_6)\text{N}=\text{CH(CH}_2)_3\text{CHO} \quad (2.2) \]

The resulting aldehyde carrier was used for covalent attachment of the proteins.

**Cross-linking using BSA combined with covalent bonding of proteins to the preliminarily modified surface of the transducer:**

Then 5ul of a solution of urease or creatinine deiminase (1000 U/ml) and BSA (60 mg/ml) in phosphate buffer, pH 7.0, was deposited onto the modified surface together with 3ul of glutaraldehyde solution (2.5% in phosphate buffer, pH 7.0). The droplet was then thoroughly mixed and allowed to dry for 2 hour. The sensor was stored in phosphate buffer at 5°C for further use.
Chapter 3

Thermal biosensor with enzymatic reaction measurements

All enzymatic reactions calorimetric measurements using the thermal biosensor include both liquid batch and continuous flow configuration are presented in this chapter. Some sections of this chapter text are adapted from the work of Ren et al (Reference [32]).

3.1 Quartz resonator characterization & thermal biosensor calibration

3.1.1 Quartz resonator based calorimetric biosensor [32]

Historically, gravimetric measurement mode has been the primary focus of quartz resonator research for bio-chemical sensing but as it turns out quartz resonators can be used as sensitive temperature sensors as well. A major impediment in the use of temperature sensitive cut of quartz resonators for thermal bio-sensing applications is the concomitant mass loading effects that confound the results and therefore make calorimetric analysis of the results difficult. In this thesis we present a novel configuration of a quartz resonator-based thermal sensor in which the reaction chamber is physically separated and located in close proximity to a micromachined quartz resonator. The coupling of heat from the reaction chamber to the quartz resonator is achieved via radiation and conduction through ambient gas. The close proximity of a reaction chamber of a few tens to hundreds of microns and the high absorption coefficient of quartz in the 8–12 mm wavelength range render this system into a very sensitive calorimeter design. The non-contact measurement results in no mass loading effects on the quartz resonator and therefore provides clear calorimetric data [31].
In this thesis, we measure the rise in temperature in real-time using the quartz crystal resonator (QCR) located right under the freestanding reactor membrane. Figure 3.1 shows the schematic illustration of the device. Freestanding micromachined membranes of small thermal mass are very sensitive to small changes in heat and are thus capable of analyzing very small quantities of reactants and products. They also have fast response times required for the real-time study of reaction kinetics. Specificity to identify or perform a selective assay is achieved by coating the reactor with specific catalysts such as enzymes, antibodies or even single stranded oligonucleotides for DNA sequence analysis. When the analyte is exposed to such immobilized enzymes or probe DNA strands on the freestanding reaction chamber, the biochemical reaction begins and its evolution in terms of the total amount of heat generated and kinetics is proportional to the concentration of the reactants as well as the rate constants of the reaction. The applications of the presented sensor can be broadly categorized under (i) health care, (ii) monitoring and control of industrial processes, and (iii) environmental monitoring.

Quartz resonators fabricated from certain crystal cuts can be used as sensitive temperature sensors with unprecedented resolutions of up to $10^{-6}$ °C. This phenomenological sensitivity of quartz crystals makes them highly competitive technology alternative to the currently used uncooled thermal detectors such as vanadium oxide-based bolometers, thermopiles, and pyroelectric detectors. Thus quartz crystal resonators can be configured as high performance thermal sensors and can achieve temperature resolutions as low as few mK.
Figure 3.1: Schematic illustration of the proposed biosensor with a separated reaction chamber placed in close proximity to the quartz resonator temperature sensor [32].

The resonance frequency of bulk acoustic wave quartz resonators is determined by the thickness of the resonator \( t \) as:

\[
f_0 = \frac{1}{2t} \sqrt{\frac{\mu}{\rho}}
\]

(3.1)

where \( m \) is the elastic modulus and \( r \) is the density of quartz. For quartz, \( \mu = 2.95 \times 10^{10} \) Pa and \( \rho = 2.65 \times 10^3 \) kg/m\(^3\). Typical commercially available resonator crystals consist of 100 to 300 mm thick quartz with resonance frequencies in the 5 to 20 MHz range. Using
bulk micromachining techniques, we have fabricated resonators from 18 mm thick Y-cut quartz. These resonators have fundamental resonance frequencies of ~90 MHz. Y-cut quartz has a temperature sensitivity of +90 ppm/K yielding a temperature sensitivity of ~8 kHz/K.

Figure 3.2: Optical photographs of the Y-cut quartz based bulk acoustic wave resonators. (a) Photograph of the sensor array with 8 pixels arranged around the perimeter of a square, (b) zoomed image of one of the pixels, and (c) packaged device on a 300 mm thick stainless steel plate. (d) Packaged device with the PDMS reaction chamber. The central hole exposes the sensor array to the reaction chamber which is placed directly atop [32].
Figure 3.2 shows an optical picture of the quartz resonator-based temperature sensor array consisting of eight 1 mm diameter and 18 mm thick micromachined Y-cut quartz resonator pixels with a resonance frequency of \(~90\) MHz and quality factor \(>10\ 000\). The fabricated sensor array is packaged onto a 300 mm thick stainless steel plate with an open window facing the sensors. A 6 x 6 mm\(^2\) biochemical reaction chamber is made by attaching PDMS mold onto a 160 mm thick glass cover slip and placed atop the sensor array. The thick PDMS walls provide good thermal isolation while the bottom glass plate couples the heat from the reaction chamber efficiently to the sensor array. The fabrication process of the quartz sensor array is described in detail elsewhere. With the sensor array mounted under the steel plate and the reaction chamber placed atop the steel plate, the gap is determined by the thickness of the plate.

3.1.2 Resonance tracking and calibration of quartz resonator [32]

The resonance frequency of the fabricated micromachined quartz resonators was monitored using an Agilent 4294A impedance analyser. The real and imaginary impedance components were measured centred around the resonance frequency in a bandwidth of 100 kHz. Data were recorded for 801 frequency points within the bandwidth. The temperature sensitivity of the resonators was measured around room temperature by placing the packaged resonator in an oven and allowing for the temperature to stabilize for 30 minutes before taking the resonance frequency measurement.
As mentioned earlier, the analyzer used in this work is capable of acquiring up to 801 impedance measurements over the experimentally set frequency span. Even using extremely fast scan rates, each impedance scan takes around 3 s. Since the typical biochemical reaction times are expected to be much faster, this method of tracking the resonance frequency is unsuitable for the current application. To overcome this limitation, the impedance analyser is set to a fixed frequency at the midpoint between the two inflexion points in the imaginary component of the impedance curve shown in Figure 3.3 and Figure 3.4, and the change in imaginary part of impedance is monitored in real-time.

![Impedance curve](image)

Figure 3.3: Impedance of the micromachined quartz resonator spanning the resonance frequency shows a very high Q-factor of >10,000 for the 88 MHz devices [32].
Figure 3.4: Monitoring the magnitude of the impedance of the resonator near its resonance at a fixed frequency operating point as the incident infrared radiation is modulated results in a modulation of the impedance [32].

For small temperature changes, an increase and/or decrease in the frequency, about the set quiescent frequency, translates into an increase or decrease in the value of $\text{Im}(Z)$. This method offers an additional gain to the measurement of temperature (T) sensitivity of resonance frequency ($f_0$) since:

$$\frac{d(\text{Im}(Z))}{dT} = \frac{d(\text{Im}(Z))}{df_0} |_{f_0} \frac{df_0}{dT} = -\text{slope} \times \frac{df_0}{dT}$$

(3.2)
Furthermore, using this method, continuous tracking of the resonance frequency reduces to real-time monitoring of the imaginary component of impedance at a fixed frequency. So long as the quiescent point of the circuit is set to be at the midpoint between the inflection points in the impedance curve, and the temperature changes are small, this method offers a very simple electronic measurement method which can be readily scaled to large arrays. Since the temperature change induced in the sensor pixel in the described biosensors is expected to be small, this method is reasonably well suited for this application. Labview based program was developed to find the maximum slope factor in the linear region of impedance vs. frequency curve and to set-up the measurement frequency for real-time measurement of admittance change during the biochemical reaction at the set maximum slope point. With this new test method, the scanning time for every data point is only ~0.15 second, which resulted in 20 times more data points being recorded during experiments.

Figure 3.5: The temperature sensitivity of the resonance frequency while calibrating the sensor in the oven [32].
Figure 3.5 shows the sensor’s temperature sensitivity by heating it in the oven from room temperature 22 °C to 38 °C, which is quite agreeable with the Y-cut quartz resonator’s resonant frequency and its temperature coefficient of frequency of 90 ppm/°C.

3.2 Liquid batch open air testing [32]

Figure 3.6 shows the schematic of the noncontact set-up used for liquid batch enzymatic sensing. These measurements are accomplished via an open chamber where the enzyme-analyte reaction occurs directly above and in close proximity to the thermal sensor as illustrated in Figure 3.6.

![Schematic illustration of the biosensor with a separated reaction chamber placed in close proximity to the quartz resonator temperature sensor. Entire set-up is placed in an oven at 37 ± 0.1 °C for creatinine concentration measurements [33].](image)

Systematic experimental studies have been performed to test the fabricated quartz resonator sensor for enthalpy measurements using liquid batch testing. The studied experiments include the neutralization reaction between hydrochloric acid and
ammonium hydroxide (from J. T. Baker), the hydrolysis of urea (from J. T. Baker) with urease (5 U/mg, EM Science Inc), and the catalytic reaction of D-(-)-glucose (99.5%) by glucose dehydrogenase (279 U/mg, Sigma Aldrich), and the catalytic reaction of creatinine (anhydrous, ≥98%) by creatinine iminohydrolase (creatinine deiminase, >25U/mg, Sigma Aldrich). For ease of operation, all the experiments are performed in open chamber condition. A reaction chamber made using a PDMS mold attached to a 160 mm thick glass or polystyrene cover slip to form a 6 x 6 mm$^2$ area reaction chamber was used and placed atop the hole in a 300 mm thick steel plate under which the quartz sensor was mounted.

3.2.1 Acid–base neutralization reaction [32]

The HCl and NH$_4$OH solutions were made by diluting the original stock solutions from the manufacturer with DI water in the range of 0.5% to 4% (wt%), respectively. Prior to the test, the sensor was connected to an Agilent impedance analyzer to stabilize for 30 minutes. The reaction chamber (opening in the PDMS walls) was aligned on top of the sensor array and 100 µl of 1% HCl solution was placed in the reaction chamber. The chamber with HCl solution was allowed to equilibrate in the ambient conditions for ~5 minutes to eliminate any temperature difference between the reaction chamber and the sensor. 100 µl of 0.5% ammonium hydroxide solution was thereafter added to the reaction chamber using a pipette and the impedance change in real-time was measured using the Labview program as described earlier. An identical procedure was repeated for 1%, 2%, 3%, and 4% NH$_4$OH solution reaction with 1% HCl solution.
The neutralization reaction can be expressed as

\[
\text{HCl} + \text{NH}_4\text{OH} \quad \xrightarrow{\text{51.5 kJ/mol}} \quad \text{NH}_4\text{Cl} + \text{H}_2\text{O}
\]
Figure 3.7: Shows the measurement of the heat of reaction of HCl-NH$_4$OH reaction using (a) Impedance tracking method (b) Frequency tracking method, (c) Comparison of the peak outputs using the two methods [31].

The enthalpy of the reaction $\Delta H = -51.5 \text{ kJ/mol}$. Figure 3.7 shows the time dependence of the output of quartz resonator sensor from the reaction of 1% HCl with various concentrations of NH$_4$OH. Since the chemical reaction requires 1:1 molar concentration for the maximum evolution of heat, the amount of heat evolved beyond 2% weight concentration of NH$_4$OH for fixed 1% HCl solution saturates. As a confirmation, a linear
response is observed if 1:1 wt% concentrations of HCl and NH₄OH are reacted. An exponential rise in the temperature with an average time-constant of 0.5 s is observed.

3.2.2 Urea sensing [32]

Figure 3.8: Output of the sensor for various concentrations of urea catalyzed by the enzyme urease. Increasing the concentration of urea results in a linear increase in the peak impedance [32].

0.05 M, 0.1, 0.2, 0.3 and 0.4M solutions of urea were prepared in phosphate buffer saline (PBS, pH 7.0) solution. Since 1 unit of urease can hydrolyze 0.5 µmol of urea, 250 U/ml urease solution in PBS was prepared which has sufficient enzymes to react with urea for all the concentrations in the experiment. Initially, 200 µl of 0.05 M urea solution was
placed into the reaction chamber. Then similar to the experimental procedure described earlier, the reaction chamber was stabilized in air for ~5 minutes following which 30 µl of urease solution was added into the urea solution in the reaction chamber. The data were acquired by the Labview program, for 0.05 M, 0.1 M, 0.2 M, 0.3 M and 0.4 M urea solutions, respectively.

The hydrolysis of urea in the presence of the catalyst urease can be written as follows:

\[
\text{CO(NH}_2\text{)}_2 + 2\text{H}_2\text{O \xrightarrow{\text{Urease } \sim 61\text{kJ/mol}}} 2\text{NH}_4^+ + \text{CO}_3^{2+} \quad (3.4)
\]

The enthalpy change of the reaction is - 61 kJ/mol. The experiment data for hydrolysis of urea are shown in Figure 3.8. The data exhibit a linear dependence of the peak sensor output as a function of the concentration of urea.

3.2.3 Glucose sensing [32]

The identical experimental procedure as described for the urea reaction was used for testing of glucose dehydrogenase and with 100 µl of glucose solutions. As it is known that 1 unit of glucose dehydrogenase enzyme can catalyze 1 µmol of D-glucose, appropriate amounts of the enzyme were diluted in phosphate buffer solution prior to the experiment. As a further demonstration of the sensor for enzymatic reaction monitoring, 1 mM, 5 mM, 10 mM and 50 mM D-glucose solutions in PBS were catalyzed using the enzyme glucose dehydrogenase.

Glucose dehydrogenase catalyses glucose according to the chemical reaction:
D-glucose + NAD (P)$^+$ \rightarrow$ D$-glucose-1,5$-$lactone + NAD (P)H$^+$ + H$^+$ \hspace{1cm} (3.5)

Figure 3.9: Output of the sensor for various concentrations of glucose catalyzed by glucose dehydrogenase enzyme. Increasing concentrations of glucose results in monotonic increase in impedance which has been fitted by a straight line [32].

This oxido-reductase enzyme acts on the CH–OH group of glucose with NAD$^+$ or NADP$^+$ as acceptor with an enthalpy of reaction of \( \sim 80 \) kJ mol$^{-1}$. Figure 3.9 shows the results of enzymatic catalysis of D-glucose using glucose dehydrogenase. For the concentrations tested, a linear dependence of the sensor output to the concentration of the glucose solution is observed. Based on the stability of the sensor output and the impedance noise, a signal to noise ratio of \( \sim 130 \) was obtained for 1 mM glucose solution allowing for a glucose detection resolution of less than 10 \( \mu \)M of D-glucose using this
technique. This resolution represents ~250 times improvement in the measurement of glucose over thermopile based sensing reported elsewhere. In normal human beings the blood glucose levels vary between 4 and 6 mM. The most accurate meter today has only 63% acceptable values at accuracies of >95%. It is in this context that the resolution of 10 μM that can be obtained using the current sensor configuration needs to be seen.

3.2.4 Creatinine sensing [33]

Figure 3.10: The output of the sensor plotted as a function of creatinine concentration on a Log-Linear scale shows a linear dependence. Inset: Realtime resonance frequency shift upon addition of 100 μl of creatinine solution to 100 μl creatinine iminohydrolase solution in phosphate buffer solution [33].
Creatinine iminohydrolase (creatinine deiminase) was prepared in phosphate buffer at a concentration of 10 Units per 100 µl. 100 µl of the enzyme solution was placed in the reaction chamber and allowed to stabilize to 37 °C. Once the device had stabilized, 100 µl of creatinine of a given concentration also stabilized in temperature in the same oven at 37 °C was pumped using a syringe pump. The pumping speed was set up at a fast rate to result in instantaneous addition of the entire volume of 100 µl of creatinine solution to the enzyme solution.

Creatinine deiminase catalyses creatinine according to the chemical reaction:

\[ \text{Creatinine} + \text{H}_2\text{O} + \text{H}^+ \rightarrow \text{N-Methylhydantoin} + \text{NH}_4^+ \]  \hspace{1cm} (3.6)

The output of the sensor plotted as a function of creatinine concentration on a Log-Linear scale shows a linear dependence and is shown in Figure 3.10. Inset in Figure 3.10 shows the real-time output of the sensor for various concentrations of creatinine. An unambiguous output is seen even at a concentration as low as 100 µM.

### 3.3 Continuous flow set-up measurements [33]

For continuous flow measurements, we have constructed a fluidic fixture made from Teflon and was sealed using a 25 µm thick Kapton® film on which urease enzyme was immobilized using glutaraldehyde as cross-linker molecule.

Figure 3.11 shows the schematic and optical pictures of the setup. The Kapton® film is squeezed between an O-ring on the Teflon fixture and the steel plate thus forming a
fluidic seal. The quartz sensor is mounted on the other side of the steel plate and directly faces the Kapton film with the immobilized enzyme through a circular hole cut out in the steel plate.

Figure 3.11: (a) Schematic illustration of the flow set-up, (b) Shows the experimental set-up with syringe pump and impedance analyzer in the background, (c, d, e) Shows the Teflon® flow channel fixture, Kapton® film with immobilized enzyme, and the packaged sensor mounted on the steel plate [33].
Figure 3.12: Sensor response to hydrolysis urea catalyzed by immobilized urease in a fluidic channel. The signal to noise ratio in the flow set-up is 50 times lower than in open chamber output and needs further optimization of the enzyme and fluidic set-up [33].

With the implemented immobilization described in the previous chapter, the functionalized Kaptop film was assembled onto the fluidic set up (Figure 3.11). The results of the tests for various concentrations of urea are shown in Figure 3.12. In each case, a measured volume of urea was pumped into the sensor chamber. The signal to noise ratio of the sensor under continuous flow testing using immobilized enzyme was ~50 times smaller than the output obtained using open chamber experiments. Results obtained thus far are highly encouraging and clearly demonstrate the feasibility of the proposed sensor for continuous urine creatinine measurements.
Chapter 4

Conclusion and future works

This chapter will summarized all works that have been carried out in this thesis. All potential future works for thermal biosensor improvement has also been discussed here.

4.1 Summary

This thesis describes a novel design and implementation of a calorimetric biosensor based on the micromachined Y-cut quartz resonator. The sensor was packaged onto a 300 mm thick stainless plate with an opening in the middle. The sensor array was aligned to the opening and mounted from the underside of the plate.

For liquid batch open air testing, a reaction chamber designed for performing biochemical reactions made from a glass or polystyrene coverslip with thick PDMS walls was specifically designed and used in the measurements. Since the reaction chamber was simply placed atop the sensor array, upon completion of the reaction they could be readily taken off and replaced with a new one for the next sensor test. This configuration of the sensor allows for a very robust sensing platform with no fouling of the sensor surface or degradation in the performance metrics. Using the recently described method for tracking resonance measurement in real-time, impedance based chemical, enzymatic measurements were made. The sensor described has an ideal impedance sensitivity of 852 \( \mu \text{V/}^\circ\text{C} \) or a frequency sensitivity of 7.32 kHz \( /^\circ\text{C} \) for the 91 MHz resonator used in this work. Several experiments were conducted to test the quartz resonator sensor, such as the exothermic reaction between hydrochloric acid and ammonium hydroxide, the hydrolysis
reaction of urea with urease and the catalytic reaction of glucose with glucose dehydrogenase.

For the continuous flow set-up configuration, the concept of a high-sensitivity, calorimetric, point-of-care device for automated bedside urine testing based on a micromachined thermal sensor is presented. Immobilized urease enzyme on thin (25 μm) Kapton® films are used to measure the catalytic calorimetric output as a function of concentration in a flow set-up and creatinine iminohydrolase (creatinine deiminase) was used to catalyze creatinine in open air batch testing set-up. The sensor design locates the active Kapton® film in close proximity to the quartz temperature sensor thereby providing an efficient heat coupling between the two. Measurements of urea concentration at room temperature using the continuous flow set-up and creatinine in open chamber set-up at 37 °C are presented.

Concentrations of 50 mM for urea and 100 μM for creatinine have been measured. This thesis presents the first non-contact continuous flow thermal sensing measurements. The unique sensor design allows for enzyme immobilized Kapton® film as an easy to swap disposable cartridge and represents a significant advancement for clinical diagnostic application.
4.2 Future works

The sensor shows good repeatability, high sensitivity and robust performance. And although single sensor measurements are reported in this work, the sensor configuration can be readily extended for array level measurements. That means each sensor pixel will be able to measure the generated heat from one type of various enzymatic reactions with the implementation of miniature reaction chambers on top of all sensor pixels. The possibility of detecting various analytes by using different immobilized enzyme cartridges is promising.

Furthermore, the results that we obtained thus far are highly encouraging and clearly demonstrate the feasibility of the proposed sensor for continuous urine creatinine measurements. With an integrated miniature fluidic system, and a special designed thermal box to create a $37^\circ$C stable temperature environment for creatinine enzymatic reaction, an automatic system of continuous flow set-up measurement for real urine sample will have a real potential.
References


[13] Webpage, Enzyme Technology - Calorimetric biosensors:


